PRODUCTION AND BIOASSAY OF *CURVULARIA PALLESCENS* BOEDIJN TOXINS

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ABSTRACT. — Studies on *in vitro* production of toxic substances by *Curvularia pallescens* Boed. was carried out using a modified Czapek's yeast extract culture medium. Properties of toxic substances produced were determined and bioassay carried out : yellow in colour, melting point 232°C, chromatography RF value 0.84, thermostable and more soluble in most organic solvents such as acetone, methanol, ethanol and chloroform than in water. Bioassay of the purified toxic substances of *C. pallescens* on 14 days old maize plants gave chlorotic spots which later became necrotic similar to *in vivo* symptoms of the disease. Roots of maize seedlings were retarded in growth on treatment with an aqueous solution of the toxin. The lowest quantity of the toxic substances that could cause plant reaction was 3.6 ± 0.1 µg. When tested with some weeds and crop plants, these toxic substances were found to be host-specific, since they did not induce similar reactions.

RÉSUMÉ. — Des études sur la production *in vitro* de toxines par *Curvularia pallescens* Boed. furent entreprises sur milieu Czapek modifié. Les propriétés et l'activité biologique des substances toxiques produites sont déterminées : couleur jaune, point de fusion de 232°C, RF de 0,84 en chromatographie, thermostable et meilleure solubilité dans la plupart des solvants organiques tels que l'acétone, le méthanol, l'éthanol et le chloroforme, que dans l'eau. L'activité biologique des substances toxiques du *C. pallescens* sur un plant de maïs de 14 jours (cultivar Igbira) produits des lésions chlorotiques qui deviennent nécrotiques, semblables aux symptômes *in vivo* de la maladie. La croissance des racines de jeunes pousses de maïs est retardée par leur traitement avec une solution aqueuse des substances toxiques. La plus faible quantité pouvant provoquer une réaction chez la plante est 3.6 ± 0.1 µg. On constate que les substances toxiques, testées sur des herbes et des plantes de culture, sont spécifiques.

KEY WORDS : *Curvularia pallescens*, toxins.

INTRODUCTION

Several pathogenic fungi are known to produce toxins which can cause similar symptoms on the host plants (MACRI & VIANELLO, 1976; NAEF-ROTH, 1972). However, some workers have dealt with several factors affecting toxin production and came out with some general basis for toxin production by

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Source : MNHN, Paris

most pathogenic fungi. For instance, *Fusarium culmorum* could only produce toxins in shake cultures at 27°C in 2-3 days, whereas *Alternaria tenuis* does so in still culture (SAAD & al., 1970). Also, DIENER & DAVIES (1969) found that sucrose, arabinose and glucose are good carbon sources for toxin production while fructose, xylose and maltose are very poor in this regard. Ammonium nitrogen was found to be the best nitrogen source for toxin production, and it has been exemplified with *Fusarium* sp. and *F. amygdali* as reported by BRIAN & al., (1961) and NAEF-ROTH (1972), respectively. For toxin production by most pathogenic fungi, high yield can be obtained from a medium augmented with corn steep liquor, casamino acid, yeast extract or peptone and some metal ions such as Cu^{++} and Mg^{++} (DIENER & DAVIS, 1969; NAEF-ROTH, 1972).

However, combinations of the above and other available information will help in preparing a suitable medium for toxin production in this study.

This study thus examines only the possibility of toxin production by *Curvularia pallescens*, a subject which has not yet been investigated although the existence of this fungus has been reported in 1969 by MABADEJE in Nigeria. The effect of different factors on toxin production will be another line of interest in another study.

MATERIALS AND METHODS

Forty out of eighty 250 ml Erlenmeyer falsks each containing 25 ml of autoclaved liquid medium were inoculated with disks of 0.4 cm diameter from 12 days old sporulating cultures of *C. pallescens* grown on potato dextrose agar (PDA). The other 40 were treated as above with uninoculated PDA to serve as control. The inoculated flasks were incubated at 25°C on an orbital shaker with 48 revolutions per minute. The liquid medium used was a slight modification of Czapek yeast extract medium.

Samples of liquid culture medium were removed, strained through cheesecloth, centrifuged and millipore-filtered. The culture filtrate was then reduced to 1/20th of its original volume by evaporation *in vacuo* at 45°C. An equal volume of methanol was added, and after 24 hours, water was separated and discarded. Methanol was evaporated from the solution to leave crystals of the toxins.

The yellow crystals obtained were further purified by running its methanol eluted solution through silica gel plates (20 x 20 x 0.2 cm) using ethyl acetate-methanol (4/7, v/v) as running solvent for the chromatography. Methanol extracts of the control flasks were also run in the same way.

Properties and bioassay of the toxic substances were determined as follows :

Melting point : This was determined by collecting the crystals in a capillary tube sealed at one end. The capillary tube was attached to the bulb end of a thermometer with a rubber band and both were immersed in paraffin oil in a container placed on a bunsen flame.

Source : MNHN, Paris

Solubility : Solubility was determined by dissolving a known quantity of the isolated crystals into some solvents (water, acetone, methanol, chloroform and ethyl acetate) at 5°C interval from 25-60°C. The maximum amount that could dissolve in the solvent until it attains the saturation point at a particular temperature was recorded.

Absorption spectrum : This spectrum was determined with the help of a Pye Unicam Spectrophotometer Sp 1800 using a 0.1/10ml solution. The length of the spectrophotometer was run through ultraviolet to the visible-infra red regions and the absorbance was recorded at intervals of 10 mn.

In vivo secretion of the toxin : Filtered methanol (20 ml) extracts of 10 g leaf spot diseased tissue were run on thin layer silica gel chromatography using the same running solvent as in the *in vitro* experiments. Comparisons were made with toxic substances isolated *in vitro*. Bioassay with the extracted toxic substances was also carried out in a way similar to the *in vitro* investigation.

Bioassay : Serial dilutions of 10^{-1}, 10^{-2} to 10^{-6} of the toxic substances were made using water (10^{-1} = 1 g/10 ml to 10^{-6} = 1 µg/ml).

Two groups were formed for the bioassay, each treatment was tested with a heated solution of toxic substances (100°C) and unheated lots, in order to assess the thermostability of the toxins.

The bioassay was carried out with shoot, excised leaves as well as the seedling roots of maize cultivar Igbira :

– The study on shoots was carried out with stem cuttings (cut under water about 10 mm above the roots), of 2 weeks old maize seedlings. Cuts ends were then quickly placed in a medium-sized graduated 20 ml specimen tube containing 10 ml of various dilutions of the toxin solution. The shoots were supported with cotton wool and wrapped with aluminium foil to prevent evaporation. Distilled water and blank culture medium were separately used for the control. Five replicates were made for each treatment.

– For excised maize leaves, 0.05ml of the toxin solution was introduced with a sterile microsyringe through the mid-rib of the leaves of size 3 x 5 cm. The control liquids were treated in the same way before the leaves were incubated at 28°C.

– For the roots, they were measured before and after the bioassay to detect any difference in length.

After obtaining the lowest amount that can cause plant reaction, the toxin solution was applied to some of the weeds found in and around maize fields. Weeds used were *Tridax procumbens*, *Eleusine indica*, *Dactylon* sp. and some crops such as *Vigna unguiculata* and *Ablemoscus esculentum*.

RESULTS

The toxic substances isolated have a light yellow colour with a melting point of 232°C. The chromatography RF was 0.84 and it was observed that the

Source : MNHN, Paris

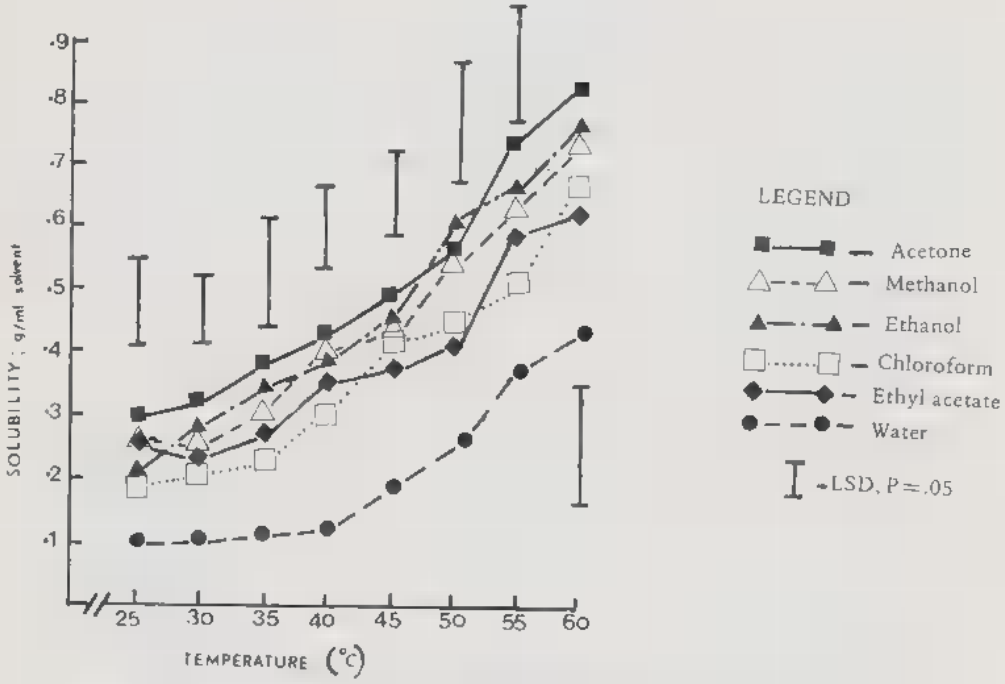

Figure 1. — Solubility curves of *Curvularia pallescens* toxins.
Figure 1. — Courbes de solubilité des substances toxiques du *Curvularia pallescens*.

toxic substances were most soluble in acetone, among the organic solvents used, and were least soluble in water (Figure 1). Solubilities in all other organic solvents were relatively similar and also greater than in water. At room temperature (25°C) acetone could dissolve 0.30 g/ml while water could only dissolve 0.10g/ml. It was observed that the peak absorbance of the toxin solution was at 420 nm.

The methanol extract of the infested tissue of maize leaves, obtained during the extraction process which was run along with the ones obtained in culture, gave similar results. The eluate of yellow band, corresponding to the reference, was observed to have identical physical properties as those of the toxic substances from culture filtrates of *C. pallescens*. The absorption spectrum showed a peak at 420 nm and the melting point was 232°C. The toxic substances were also found to be moderately soluble in water and highly soluble in most organic solvents such as acetone, methanol, ethyl acetate and chloroform. The toxic substances were produced both in culture and in infected plants.

Results on bioassay given in Table 1 showed that dilutions of 10^{-1} to 10^{-3} induced observable reactions 25 hrs after the immersion of maize seedlings cut ends in the toxin solution. Results also showed that 0.26 and 0.28 ml of the

Source : MNHN, Paris

Table 1 - Bioassay of *Curvularia pallescens* toxins. Volume of solutions imbibed (ml) by the cut ends of maize seedlings that cause plant reaction, in relation to time of incubation and concentration of the toxic substances.

Tableau 1 – Activité biologique des substances toxiques du *Curvularia pallescens*. Volume des solutions imbibées (ml) par les semences de maïs, provoquant la réaction de la plante, en fonction du temps d'incubation et de la concentration en substances toxiques.

Time	TOXIC SUBSTANCES						CONTROL	
(hrs incubation)	serial dilutions (10^{-6} = 1 µg/ml)						Blank medium	dist. water
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}		
25	0.26*	0.28*	0.28*	–	–	–	–	–
29	0.30*	0.31*	0.33*	0.34*	–	–	–	–
56	0.33*	0.34*	0.35*	0.35*	0.36*	–	–	–

Nota : at 76 hrs, all the testing plants show general chlorosis, even the controls.
* = Plant reaction; – = no plant reaction.

respective dilutions were imbibed to produce reactions in plants. Plants started to react to the 10^{-4} dilution after 29 hrs during which 0.34 ml of toxin solution was imbibed. With the 10^{-5} dilution, plants reacted after 56 hrs during which 0.36 ml of the toxin was imbibed. The 10^{-6} dilution did not show any reaction. After 76 hrs, all the plants became chlorotic, even the control.

Also, on Table 1 it was evident from the bioassay of dilution 10^{-5} that 3.6 µg was the lowest quantity that can cause observable reaction in 2 weeks old maize plant leaves. Thus, 10^{-6} dilution which contained 1.0 µg/ml, had to reach a quantity of toxic substances of 3.6 µg in the plants before it can cause reaction, and that might not be attained before physiological chlorosis set in.

The general plant reaction was initiated by pin-point chlorotic spots or patches in some cases. This later developed into circular ovoid straw coloured or brownish spots surrounded by chlorotic halo. This was not noticed on the control plants.

Table 2 – Reaction of maize leaves to *Curvularia pallescens* toxin after 25 hours of incubation.

Tableau 2 – Réaction des feuilles de maïs à la toxine du *Curvularia pallescens* après 25 heures d'incubation.

Volume	TOXIN SOLUTION						CONTROL	
Leaves	serial dilutions (10^{-6} = 1 µg/ml)						Blank medium	dist. water
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}		
0.05 ml	+	+	+	+	–	–	–	–

* = Plant reaction; – = no plant reaction.

Source : MNHN, Paris

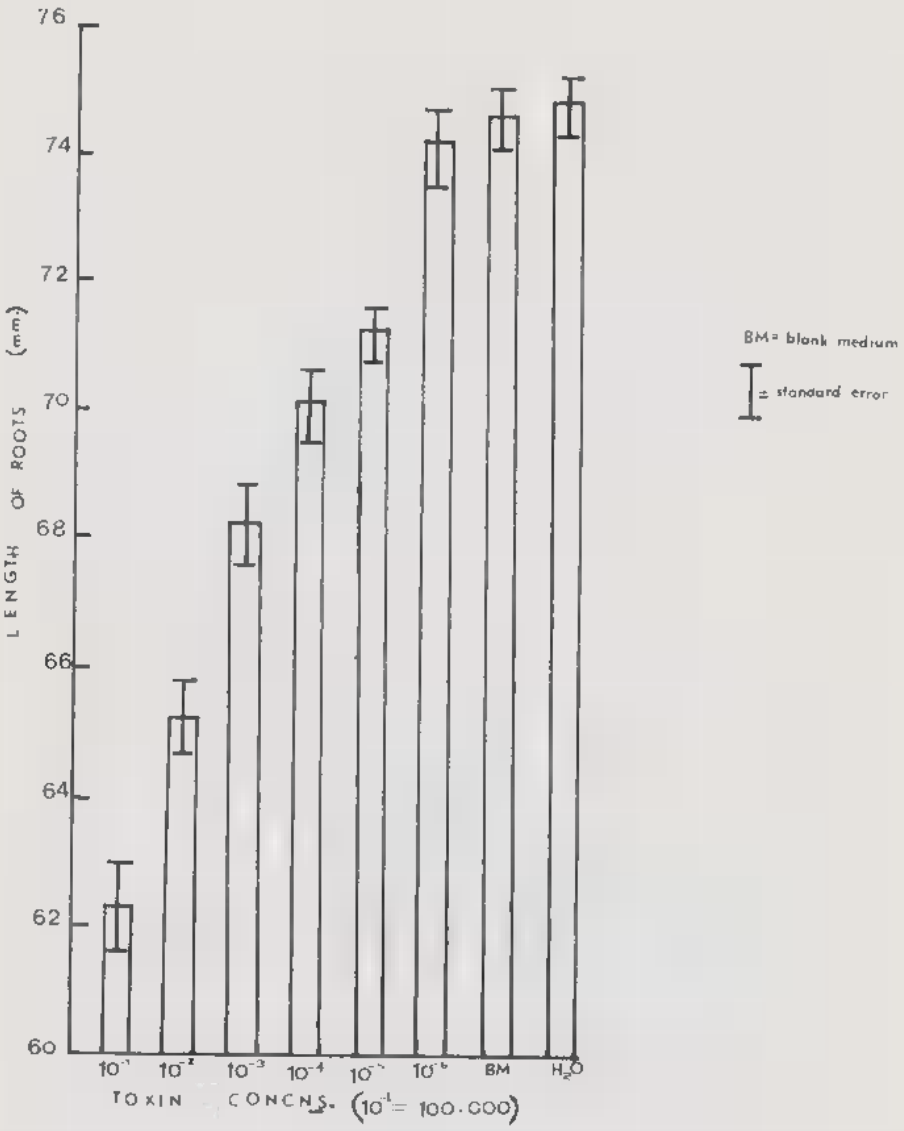

Figure 2. — Bioassay of *Curvularia pallescens* toxins using maize seedlings roots.

Figure 2. — Activité biologique des substances toxiques du *Curvularia pallescens* sur racines de semences de maïs.

From the excised leaf experiment (Table 2), it was also observed that 5.0 µg was the lowest amount to cause plant reaction, due to the static volume of the toxin used among the various dilutions (i. e. 0.05 ml). However, in the other two assay methods the plant suck the solution itself.

Source : MNHN, Paris

With the seedlings root bioassay, increase in toxin concentration retarded elongation of the seedlings roots significantly up to dilution 10^{-5}. Compared with blank culture medium and distilled water, dilution 10^{-6} did not significantly retards roots elongation. There was a significant difference between dilutions 10^{-5} and 10^{-6} (Figure 2).

Finally, none of the weeds and the crop plants used for bioassay showed observable symptoms similar to those developed by maize plants; they behave as if nothing was added to their water solution.

DISCUSSION

The results of the bioassay indicates the presence of toxic substances in the culture filtrate of *C. pallescens*. This conforms with the work of MACRI & VIANELLO (1976) when they worked on *Curvularia lunata* (Wakk) Boed.

The presence of toxin could also be reaffirmed by the fact that similar symptoms were obtained when plants were inoculated with a solution of the toxin and with fungal spores. The toxic substances could be termed thermostable since both heated and unheated solutions gave the same reactions.

The bioassay of toxin serial dilutions showed that at any concentration, it would take up to 25 hrs for the toxins to get the plant cells and cause reactions. From dilutions 10^{-5}, it could be deduced that 3.6 ± 0.1 µg was the lowest possible amount that could be taken into the host tissue to cause plant reaction.

In the excised leaf bioassay, it could be observed that the amount needed to cause plant reaction using a static volume of the toxin was 5.0 µg.

The inability of dilution 10^{-6} as well as the control to cause root growth retardation could be due to the fact that the required toxin quantity was not attained, till the termination of the bioassay; PRINGLE & SCHEFFER (1967) observed similar type of retardation in root elongation caused by *H. carbonum* toxin on maize seedlings.

According to SCHEFFER & YODER (1972), and BHULLAR & al. (1975), leaf chlorosis and necrosis symptoms caused by toxins affect leaf cells plastids, in that their chloroplasts were disorganised and reduced in quantity, which in turn reduced leaf photosynthesis. In this study, the chlorosis and necrosis were obtained, and one could infer that photosynthesis would also be reduced.

The shoot experiments are the best bioassay method of the toxins due to the following reasons. First, out of the three methods, it was the best for determining the lowest possible concentration needed to cause plant reaction, and the volume of the solution taken could be easily detected. Second, one would need no extra measuring instrument to observe plant reaction, but in the case of using roots for bioassay, roots has to be taken before one can really know the occurrence and magnitude of growth retardation caused by the toxins.

With the test on some other weeds showing no similar symptoms and crop plants, it became evident that the toxic substances could be host-specific.

Source : MNHN, Paris

The *Curvularia pallescens* toxins have been extracted from both artificial medium and from diseased host leaves. Due to the fact that they have been purified and found to be phytotoxic, causing chlorosis and partial necrosis and increase in severity of the symptoms with the increase in toxin concentration, establishes the fact that they were obtained from *C. pallescens*. This conforms with the vivotoxicity and pathotoxicity phenomena proposed by DIMMOND & WAGGONER (1953) and WHELLER & LUKE (1965), respectively which stipulated that vivotoxin or pathotoxin obtained from a known pathogen should be able to cause the same disease as the pathogen as well as being extractable from diseased sites on the host. This has, however, been proved in this study.

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Source : MNHN, Paris